THE DIAGNOSIS OF INTESTINAL FLAGELLATES BY CULTURE METHODS

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The study of the feces of thousands of persons during the past few years has indicated infection with *Chilomastix mesnili* in about 4 per cent. of cases and with *Trichomonas hominis* in about 3 per cent. (Hegner and Payne, 1921). These results are based on specimens obtained from patients suffering from intestinal disorders, or from other diseases not involving the alimentary canal, as well as from apparently healthy persons. The methods used for determining the presence or absence of these and other flagellates have been of various sorts and of different degrees of accuracy. No one, however, seems to have used the culture method described in this paper for purposes of diagnosis.

The intestinal flagellates of man in the probable order of their importance are as follows: (1) *Giardia lamblia* Stiles 1915, (2) *Chilomastix mesnili* (Wenyon) Alexeieff 1912, (3) *Trichomonas hominis* Davaine 1860, (4) *Embadomonas intestinalis* Wenyon and O'Connor 1917, and (5) *Enteromonas hominis* da Fonseca 1915. No species of the genus *Giardia* has ever been cultivated in artificial media and hence the culture method is not now applicable to *G. lamblia*. *Chilomastix mesnili* and *Embadomonas intestinalis* were both cultivated for the first time in this laboratory by Boeck (1921) and Hogue (1921 a), respectively, and *Trichomonas hominis* has been grown in artificial media by a number of investigators (see Hogue, 1921 b). We have found flagellates in fecal cultures from two persons that resemble the descriptions of *Enteromonas hominis*, but our preparations fixed in Schaudinn's fluid and stained by Heidenhain's iron-hemotoxylin method are not sufficiently clear to use for purposes of identification.

The methods of diagnosis commonly in use are as follows:

1. A small portion of the fecal material is mixed on a slide with a drop or two of normal saline solution, and spread out under a cover glass. The movements of active flagellates render them quite conspicuous when examined with relatively low magnification. Flagellates in the trophozoite stage that have ceased to move are more difficult to find, unless very numerous, even with the high dry objective. Cyst stages are known for all of the species listed above except *Trichomonas*

hominis and these can be distinguished from one another by shape, size and contents. The diagnostic characteristics of the trophozoites and cysts of these flagellates are stated in various recent publications, such as Hegner and Cort, 1921, and Dobell and O'Connor, 1921.

2. A similar fecal sample may be mixed with a solution of 5 per cent. potassium iodid in normal salt solution saturated with iodin. Material prepared in this way is particularly valuable for bringing out the internal structure of cysts, but of course this treatment renders the free forms immobile.

3. Eosin or neutral red in solutions of 1:10,000 are useful in determining the viability of cysts since dead cysts are stained by them and living cysts are not.

4. Fixation of wet smears in hot Schaudinn's solution, followed by Heidenhain's iron-hemotoxylin stain, has been found to give the most accurate results and has been employed by some investigators in doubtful cases.

5. Concentration methods such as that of Cropper and Row, modified by Boeck (1917), may give a higher percentage of positives.

The ease with which certain of these intestinal flagellates can be grown in culture, and the fact that the movements of trophozoites cease soon after the feces are passed, suggested the experiments recorded in this paper. The culture method might be expected to give favorable results in the case of *Trichomonas hominis,* a species whose cysts, if any, have not yet been discovered, and in the diagnosis of feces that have been passed for some time and hence contain no actively motile trophozoites, or in feces that contain such a small number of passive or active trophozoites that the chances of finding one in a smear are very slight. The successful cultivation of human intestinal flagellates is of quite recent date. So far all such cultures have contained bacteria which may serve as a necessary factor in the food supply of these flagellates.

Chilomastix mesnili was grown and maintained in culture for a period of four and one-half months by Boeck in 1920. He employed most successfully a medium consisting of one part of human blood serum and four parts of Locke's solution, plus 0.25 gr. dextrose per 100 c.c. A more limited growth and reproduction was obtained when horse or sheep serum was substituted for the human serum. The culture medium was distributed in 5 c.c. amounts in small test tubes. These were incubated at 37 C. over night and those which showed bacterial contamination the next day were discarded. A loop of feces containing numerous flagellates was washed in several successive samples of warm sterile normal saline solution. A few drops of this suspension were then transferred into several tubes of culture medium and incubated at 37 C. The next day transplants were made from the

positive tubes. Most of the flagellates were found feeding upon bacterial clumps at the bottom of the tube. No cysts were found in any of the cultures.

Various authors have cultivated *Trichomonas hominis* in various media. The most pronounced success, however, seems to have been attained by Hogue (1921 b), who carried on pure line as well as general stock cultures for several weeks. One of the media used by her was made by thoroughly shaking up a hen's egg in a flask with glass beads, and adding to it 200 c.c. of Locke's solution. This was heated over a hot water bath and kept in constant motion for 15 minutes. It was then filtered through cotton with a suction pump, tubed in 6 c.c. amounts, and autoclaved for 20 minutes at 15 pounds pressure. She also employed successfully an ovomucoid medium which will be discussed later. The tubes were inoculated and incubated at 35 C. In these media the Trichomonas appeared in greatest numbers on the 2nd and 3rd days. The addition of a few drops of human or sheep serum increased the number greatly. No cysts were found in any of her cultures of Trichomonas.

Embadomonas (Waskia) intestinalis was cultivated by the same author (Hogue, 1921 a) in the 2 media which she employed for *Trichomonas hominis,* in the Boeck medium described above, and in an ox bile salt medium. Both the motile and cyst forms appeared in the cultures.

Recently Wenyon (1922) has employed a modification of Noguchi's serum medium for the cultivation of a Leptospira and various protozoa, among them *Embadomonas intestinalis.* a species of Embadomonas from the guinea-pig, and a Trichomonas from a tortoise. This medium is prepared as follows:

To 270 c.c. of 0.85 per cent. saline solution (P_H. 7.6) are added 30 c.c. of ordinary 2 per cent. bacteriologic nutrient agar (P_H 7.6). Ten c.c. of this mixture is placed in each test tube, and autoclaved at 120 C. When the tubes have cooled to 50 C., 20 drops of blood are allowed to drop into each tube from the margin of a rabbit's ear, previously shaved, sterilized with alcoholic iodine, and paraffined. After incubating for 24 hours the medium is ready for use.

This year in this laboratory we have found that *Trichomonas hominis* can easily be obtained in culture from an infected stool, and that the cultures can be carried along indefinitely in the Hogue modification of the ovomucoid medium. In addition, cultures of *Chilomastix mesnili* were readily obtained in this medium by inoculating infected material into the test tube and incubating at about 36 C. Transplants were made every two days for more than a month, and the flagellates were still growing vigorously when we ceased subculturing. We have never failed to get a heavy growth of *Trichomonas hominis* upon the first inoculation, but *Chilomastix mesnili* often appears only sparsely

in the first culture. However, upon inoculating a large amount of the first culture into a second tube of medium, swarming cultures almost always result.

A medium very similar to the Hogue formula was employed by Wherry (1913) in the cultivation of a free living amoeba. The difference between them lies chiefly in the fact that Wherry diluted the eggwhite to a somewhat less extent and used distilled water instead of normal saline solution. Wherry notes that egg white and water had been used previously by 1 or 2 authors as a culture medium for amoeba. However, the medium was first adapted to the cultivation of human intestinal flagellates by Hogue, as previously mentioned in this paper, and will be called the ovomucoid medium.

Preparation and use of the ovomucoid medium. We prepared our medium according to the simple directions given by Hogue. Briefly, the process is this: The whites of six hen's eggs are thoroughly shaken up with glass beads. This is added to 600 c.c. of 0.7 per cent. sodium chlorid solution. The mixture is cooked for 20 to 30 minutes over a boiling water bath, and is constantly agitated while cooking. The coarsest of the coagulated albumin is strained out by first passing through coarse cheese cloth. This is followed by filtering through cotton with a suction pump. The filtrate is then still quite opalescent. By means of a large pipette about 5 c.c. of this filtrate is put into each test tube, and the tubes are stoppered with cotton plugs. These are autoclaved at 15 pounds pressure for 20 minutes.

After autoclaving and allowing to stand a while, there should be a fairly clear, though slightly cloudy, supernatant fluid and a white flocculent precipitate. We have prepared this medium many times, but once such a precipitate did not form, the medium remaining colloidal. Trichomonas grew fairly well on this medium, but Chilomastix struggled along with difficulty. We should advise against using the medium for the cultivation of Chilomastix if this occurs, which is probably very seldom. If the medium is sterile it can be kept indefinitely, unless it is permitted to evaporate.

METHOD OF INOCULATION OF OVOMUCOID MEDIUM

We have found it very convenient to transfer the sample of feces to be tested for flagellates to the medium by means of a toothpick. Material is collected on the toothpick by taking a minute amount at random from different parts of the stool until an amount somewhat greater than the size of an apple seed is obtained. The toothpick is then dropped into a test tube of the medium by means of a pair of forceps, and the tube containing both fecal sample and toothpick is incubated at about 36 C.

The medium should be examined for flagellates about 24 hours after inoculation. If present they will be most numerous near the surface of

the medium. A large drop is looped off the surface onto a glass slide, and examined carefully under the low power of the microscope. We do not use a cover-glass on this drop since it spreads it out over a larger area and increases the difficulty in finding the flagellates if they are present only in small numbers. In order to determine the species of flagellate the examination should be made with the higher powers. However, if one is not quite familiar with the characteristics which distinguish the species, it is best to fix and stain cover slip smears made from the surface of the culture by the Schaudinn iron-hematoxylin method. The reader who wishes to acquaint himself with the morphology of the human intestinal flagellates should consult the papers listed at the end of this article.

In order to increase the certainty of the test for Chilomastix, it is best to pipette off the upper one-fourth of the apparently negative cultures and transfer this to a fresh tube of the medium. If Chilomastix is present it will then multiply very rapidly. No difficulty is experienced in obtaining growths of Trichomonas upon the first inoculation from a positive stool. Although we have encountered no Embadomonas (Waskia) infections, we see no reason why our method should not be useful in diagnosis of such infections, in view of Hogue's success in growing it on this medium. We found no mixed flagellate infections among the cases listed in this paper, but Chilomastix, Trichomonas, and Embadomonas have been grown in this laboratory all in a single tube in the ovomucoid medium, and media inoculated with fecal samples containing several of these species would no doubt favor the multiplication of them all, and hence aid in the diagnosis of such mixed infections.

The fact that *Trichomonas hominis* and *Chilomastix mesnili* could be so easily grown upon the ovomucoid medium suggested that we try it out on a small scale as a method of diagnosis of intestinal flagellates. The stools used were obtained through the kindness of Dr. C. B. Ensor from the Mount Hope Retreat insane hospital, from the Johns Hopkins Hospital with the assistance of Dr. C. G. Guthrie, and from a member of the school of hygiene and public health who carried an infection of *Chilomastix mesnili.*

The number of specimens examined, source of material, number found positive by the routine examination of smears, and number found positive by the culture methods are given in the following table:

TABLE 1.—COMPARATIVE RESULTS OBTAINED IN THE DIAGNOSIS OF INTESTINAL FLAGELLATES BY THE SMEAR AND CULTURE METHODS

Number of Specimens	Source of Material	No. of Positives by Smear Method	No. of Positives by Culture Method
43	Mount Hope Retreat.	0	5
67	Johns Hopkins Hosp.	2	3

Table 1 shows that the stools obtained from the Mount Hope Retreat insane hospital were all negative when examined by the smear method, but that 5 were found to be positive when inoculated into cultures. The flagellates found were 3 cases with *Trichomonas hominis* and 2 with *Enteromonas hominis* (?). Thus 5 positives out of 43 samples were overlooked by the smear method but discovered by the culture method.

It is of interest to note with respect to the material from the Johns Hopkins Hospital that in both cases in which flagellates appeared in the smears, one with *Trichomonas hominis* and the other with *Chilomastix mesnili,* they also appeared in the corresponding culture tubes.

The presence of a *Chilomastix mesnili* carrier in our department made it possible to make several examinations at intervals of the feces of a person known to be infected. Smears and cultures were made from fresh stools on the following dates and trophozoites were recorded as indicated.

Date	Smear Method	Culture Method
1. December 5, 1921.....	Positive	Positive
2. January 29, 1922.....	Negative	Positive
3. February 4, 1922.....	Negative	Positive
4. February 6, 1922.....	Negative	Negative
5. February 12, 1922.....	Negative	Negative
6. February 21, 1922.....	Positive for cysts.....	Positive
7. March 6, 1922.....	Negative	Positive
8. April 11, 1922.....	Positive	Positive
9. April 23, 1922.....	Positive	Positive
10. May 4, 1922.....	Positive	Positive

These data show that active flagellates were found by the routine smear method on 4 out of 10 examinations, but by the culture method on 8 out of 10 trials, a difference very much in favor of the culture method.

It is well known to those who have followed the course of infections with intestinal flagellates that sometimes no specimens can be found in the feces; at other times cysts only or trophozoites only are present; and sometimes both cysts and trophozoites appear in the stools of a carrier. The numbers of these, when present, likewise differ markedly at different times. The correct interpretation, therefore, of the data presented above probably is as follows. Smears were found to be positive for trophozoites on dates 1, 8, 9 and 10 because a rather large number of these were present. The smears were found negative for trophozoites on dates 2, 3, 6 and 7, because only a very few were present. Trophozoites probably appeared in the culture on date 6 because there were a few trophozoites in the sample, since the cysts

that were present are not known to develop into trophozoites in culture medium.

Efforts were made to determine how long after defecation it is possible to obtain positive cultures with *Chilomastix mesnili* and *Trichomonas hominis*. Accordingly, smear examinations of stools known to be positive for Chilomastix were made at various intervals; and cultures were made at the same time, with the following results:

Chilomastix mesnili

Time Stool Was Passed	Time of Examination and of Culture	Results of Smear Examination	Results of Culture
May 4, 7:30 p. m..	May 4, 7:30 p. m..	Positive	Positive
May 4, 7:30 p. m..	May 5, 8:00 a. m..	Positive—few alive..	Positive
May 4, 7:30 p. m..	May 5, 1:30 p. m..	Negative	Positive
May 4, 7:30 p. m..	May 5, 3:30 p. m..	Negative	Negative

These results show that living trophozoites of *Chilomastix mesnili* may appear in cultures after they can no longer be found in the stools by the smear method. Trophozoites of this species were not found in smears after 12½ hours but appear in cultures after 18 hours.

Several other experiments indicate that the trophozoites of Chilomastix remain alive in stools kept at room temperature for less than 24 hours. For example: (1) fecal material from a stool passed on Jan. 29, 1922, was inoculated into tubes immediately which later contained trophozoites. Other tubes inoculated when the stool was 24 and 48 hours old, respectively, were negative. (2) Another stool containing trophozoites was obtained on April 23, 1922, at 7:10 p. m. Cultures inoculated at 8 p. m. on April 23, and at 8 a. m., 10 a. m., 12 n., and 3 p. m. on April 24 were all positive, thus showing that these organisms were still viable 19 hours after defecation. Cultures made later were all negative.

One of our experiments indicates that the trophozoites of Chilomastix remain alive longer in fecal material kept at room temperature than in fecal material placed in an incubator heated to 37 C. A stool containing both active flagellates and cysts was obtained on April 11 and divided into 2 portions, 1 portion being placed in a moist chamber at room temperature (27 C.) and the other in the incubator at 37 C. Cultures from these were made 12 hours later; those inoculated with material that had been in the incubator were negative, the others, inoculated with the material kept at room temperature, were positive.

Smear examinations were made and culture tubes inoculated with material from a stool containing large numbers of *Trichomonas hominis* kept in a moist chamber. The results of this study are as follows:

Trichomonas hominis

Time Stool Was Passed	Time of Smear Examination and of Culture	Results of Smear Examination	Results of Culture
April 29, 9:00 a. m..	April 29, 12:15 p. m.	Positive	Positive
April 29, 9:00 a. m..	April 29, 11:00 p. m.	Positive	Positive
April 29, 9:00 a. m..	April 30, 12:00 N..	Positive	Positive
April 29, 9:00 a. m..	April 30, 10:30 p. m.	Positive (few).....	Positive
April 29, 9:00 a. m..	May 1, 8:00 a. m..	Negative	Positive
April 29, 9:00 a. m..	May 1, 9:00 p. m..	Negative	Positive
April 29, 9:00 a. m..	May 2, 7:00 a. m..	Negative	Positive
April 29, 9:00 a. m..	May 2, 4:00 p. m..	Negative	Positive
April 29, 9:00 a. m..	May 3, 8:00 a. m..	Negative	Negative
April 29, 9:00 a. m..	May 3, 4:00 p. m..	Negative	Negative

As the above table shows, no motile trophozoites were found by the smear method 47 hours after the stool was passed, but positive cultures were obtained when the feces were 79 hours old.

It will be seen from these data that *Chilomastix mesnili* is not nearly so viable in stools as is *Trichomonas hominis*. This fact may have some bearing on the apparent absence of cysts in the life cycle of the human Trichomonas, the great viability of the trophozoite of this species making it possible for it to gain access to new hosts without the aid of cysts.

Perhaps an extremely painstaking and prolonged examination, such as would not be practicable in actual diagnostic work, would have disclosed the presence of a very few organisms still alive in the specimens recorded as negative. However, the fact that they were readily cultivated from these old stools shows a decided advantage for the culture method.

Our data also furnish evidence that trophozoites of Chilomastix do not develop from cysts in the types of cultures we used since viable cysts were present in the stools at times when negative cultures were obtained.

SUMMARY

1. The trophozoites of 4 species of human intestinal flagellates may be grown in a simple culture medium; these are *Chilomastix mesnili, Trichomonas hominis, Embadomonas intestinalis,* and *Enteromonas hominis.*

2. The simplest and most practicable culture medium is the ovomucoid, consisting of a mixture of white of egg and 0.7 per cent. normal saline solution.

3. The superiority of the culture method over the smear method for diagnostic purposes is indicated by the following data: (1) Fecal specimens were obtained from 110 individuals. Trophozoites were found by the smear method in only 2 of these; whereas 8 of the culture

tubes were positive. (2) Stools from a Chilomastix carrier were examined on 10 days at various intervals between Dec. 5, 1921, and May 4, 1922. Smears from these were positive for trophozoites on 4 occasions, but the cultures contained trophozoites on 8 days. (3) Trophozoites of both Chilomastix and Trichomonas may be obtained from stools by the culture method after they can no longer be found by the smear method.

4. Trophozoites of Trichomonas appear to be more viable than those of Chilomastix. Positive smears of Trichomonas were obtained 37½ hours after a stool was passed and of Chilomastix only 12½ hours after defecation. Trichomonas was obtained in culture from a stool 79 hours old, whereas Chilomastix could be cultivated only from 18 to 19 hours after the stool was passed. It is suggested that the great viability of the trophozoite of Trichomonas may enable this species to gain access to new hosts without the aid of cysts.

5. Cysts of Chilomastix did not give rise to trophozoites in our culture media.

6. The preliminary study made of this subject lead us to conclude that routine stool examinations by the culture method are not only possible but also practicable and more efficient than those made by the smear method. The culture method is indicated, especially when the organisms are likely to be very few in number or under circumstances that make it impossible to obtain the stools for examination when fresh. It is particularly valuable for the diagnosis of *Trichomonas hominis* which has no known cyst stage to aid us in determining its presence. We hope to be able to test this method further in the near future.

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